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Cloning and Expression of Genes for Dengue Virus Type-2 Encoded-Antigens for Rapid Diagnosis and Vaccine Development

ANNUAL PROGRESS REPORT

by

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1. It is confirmed that a short peptide, QLKLNWFKKGSS can elicit neutralizing antibodies in rabbits. The titer of the antibodies in neutralization was 1:320 for a 50% reduction

2. Attempt to express NS1 in mammalian cells was made using NS1 coding sequence cloned under the control of RSV promoter. No protein was detected by in vivo labeling and immunoprecipitation techniques. The same coding sequence of NS1 when transferred to a vaccinia-based expression system which produces both the mRNA and the protein in the cytoplasm, there was good expression of NS1, suggesting that there is no lethal mutation in the coding sequence of NS1.

3. A long cDNA from nucleotide 7 to 4700 of DEN-2 genome was constructed.

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FOREWORD

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3.0 INTRODUCTION

a. General:

Dengue is a human disease caused by dengue virus, a member of the family flaviviridae (Westaway et al., 1985). Dengue viruses are of four distinct serotypes and are transmitted to humans principally by *Aedes aegypti* mosquitos. Apart from dengue fever (DF), a more severe and fatal form of the disease occurs in children called dengue hemorrhagic fever (DHF) which could lead to the shock syndrome (DSS) (for a review see Halstead, 1988). A potential problem associated with monovalent dengue vaccines is that individuals infected with one serotype are fully susceptible to infection with other serotypes resulting in a serious form of the disease, DHF, or DSS. A live attenuated vaccine was developed, but its use in volunteers was discontinued due to inconsistent neutralizing immune response among some volunteers, (Bancroft et al., 1984; Eckels et al., 1984).

Using recombinant DNA technology, it has been possible to map the antigenic determinants involved in virus neutralization, virus-cell interaction, and the proteins involved in processing, replication and virion assembly. Use of synthetic peptides encoding the epitopes of viral antigens recognized by host immune system has augmented our prospects of developing safer vaccines against a number of viral diseases. The following is the brief summary of our present knowledge of the genomic organization of flaviviruses obtained using recombinant DNA techniques.

b. Genomic organization

This group of viruses contain a single stranded RNA of about 11 kb as their genome with a positive strand polarity (Russell, et al., 1980), with a type I 5'-cap structure, but without a poly(A) tract at the 3' end. The first total flavivirus nucleotide (nt) sequence was that of yellow fever virus (YF) (Rice et al., 1985). This study established that there is a single long open reading frame coding for a large polyprotein. It was

suggested that the polyprotein is proteolytically cleaved by cellular and/or viral proteases to form the mature structural proteins, capsid (C), membrane (M), envelope (E) and nonstructural proteins, NS1, NS2A, NS2B, NS3, ns4a, NS4B, and NS5 respectively (Rice et al., 1985; Speight et al., 1988). Of the structural proteins, prM(M), E, and NS1 are glycoproteins. Their processing into mature proteins takes place in the endoplasmic reticulum (ER) by the host signal peptidase. The signal for the transport of the polyprotein precursor into ER is the presence of hydrophobic amino acid residues at the junction of the cleavage sites (Fig. 1). Since then, the complete or partial nucleotide sequences of a number of flavivirus RNA genomes have been determined: West Nile virus (WN) (Castle et al., 1985; 1986), DEN-1 (Mason et al., 1987), DEN-4 (Zhao et al., 1987; Mackow et al., 1988), PR-159 isolate of DEN-2/S1 strain (Hahn et al., 1988), DEN-2 strain 1409 isolated in Jamaica in 1983 (DEN-2JAM (Duebel et al., 1986; 1988), JE (Sumiyoshi et al., 1987), and Kunjin (Coia et al., 1988). The complete sequence of the DEN-2NGS-C genome, 10,723 nt in length with the exception of about seven nt from the 5'-noncoding region was determined in our laboratory (Yaegashi et al., 1986; Putnak et al., 1988; Irie et al., 1989). The genomic organization of all flaviviruses appears to be similar.

c. Use of recombinant DNA-based expression systems

The amount of virion proteins synthesized in the infected host cells is not sufficient to permit detailed characterization of their biological function in viral/cell interaction, neutralization and host immune response, replication and assembly of virus particles. In order to circumvent this problem, several groups have begun to utilize recombinant DNA-based high level expression systems and characterize functions of flavivirus proteins. Two notable expression systems are: 1) the recombinant vaccinia virus; 2) recombinant baculovirus. Use of these systems has shed light on some of the processing events of polyprotein precursor. For example, using a vaccinia virus vector, three glycoproteins prM, E, NS1 were produced from a cDNA fragment encoding C, prM, E, and NS1. Infection of cotton rats with the vaccinia virus recombinants induced a poor immune

response to NS1, whereas antibodies to the other two glycoproteins were not detected in the infected animals (Zhao et al., 1987). Therefore, Zhang et al. (1988) constructed a recombinant baculovirus expression vector containing DEN-4 cDNA fragment encoding C, prM, E, NS1, and NS2A. Synthesis of C, prM, E, and NS1 were detected in the infected-insect (Sf 9) cells, suggesting that the processing of the precursor protein was normal in the infected-cells. Rabbits immunized with these DEN-4 proteins developed antibodies to prM, E, and NS1, although the titers were low (1:80-1:160). Other serological assays, such as neutralization measured by plaque reduction, hemagglutination-inhibition, and complement fixation were negative. However, these antigens conferred protective immune response in mice to fatal challenge with DEN-4 virus (Zhang et al., 1988). This protection was attributed due to NS1, based on an earlier observation with YF-NS1, or with DEN-2 NS1 (Schlesinger et al., 1985; 1987). Recently, using mouse model system, protective immune response of lysates of cells infected with recombinant baculovirus and vaccinia virus expressing DEN-4 E was reported (Lai, et al., 1989; Bray et al., 1989). However, using vaccinia recombinant expressing DEN-2 E, Deubel et al. (1988) failed to observe any immune response in mice, hamsters and monkeys, as well as any protection of monkeys against viriemia with DEN-2. Some baculovirus-expressed recombinant proteins of DEN-2 E, JE-E also failed to induce protective immune response (Robert Putnak, personal communication). Further work is clearly necessary to sort out the differences between the proteins expressed in different systems in order to understand their role in protection.

Using vaccinia virus recombinant expression system, Falgout et al. (1989) studied the processing of NS1 from the polyprotein precursor. These studies revealed that the hydrophobic region present at the N-terminus of NS1 was required for the transport of the protein to ER, and for its cleavage by signal peptidase. The processing of NS1 to generate its authentic C-terminus required the full coding sequence of NS2A. This important finding raises interesting questions regarding the function of NS2A.

The variable portion of the flavivirus glycoprotein E which is the major portion of the molecule contains epitopes which determine the type specificity and complex specificity. These antigenic specificities form the basis for serological classification of flaviviruses as determined by haemaglutination-inhibition (HI) and neutralization tests. The glycoprotein E is the most important for the induction of neutralizing antibodies as well as protective immunity (Della-Porta and Westaway, 1977; Kitano et al., 1974; Heinz et al., 1981). In order to develop a subunit vaccine against dengue virus, it is important to identify the region of E glycoprotein which interacts with the host cell, or to identify its neutralizing epitopes.

Heinz et al. (1983a) presented a model for the antigenic structure of flavivirus glycoprotein E consisting of variable and conserved epitopes by analysis of glycoprotein of tick-borne encephalitis virus (TBE) (Heinz et al., 1983a; 1983b). These studies revealed the existence of three antigenic domains (A,B,and C) on E glycoprotein of TBE. Domains A & B contain neutralization-protective, and HI epitopes. The 3-dimensional structure of the E protein is important for the integrity of these determinants. Flavivirus group-reactive monoclonal antibodies although reactive in HI, were not involved in neutralization (Heinz, et al., 1983b). Heinz also noted that only those monoclonal antibodies (Mabs) which neutralized the virus in vitro also passively protected mice against lethal challenge with TBE virus.

d. Mapping epitopes.

Various methods are available to determine the precise location of antigenic sites on protein molecule (Berzofsky, 1985). One approach involves the use of Mabs as specific reagents for defining single epitopes on the complex antigenic structure of protein molecules. Using methods involving screening of purified proteolytic fragments of a protein molecule and/or screening a collection of overlapping synthetic peptides, the antigenic determinants of a number of viral glycoproteins have been studied (Roehrig et al., 1982; 1983; Lubeck and Gerhard, 1982; Emini et al., 1982; Volk et al., 1982; Massey

and Schochetman, 1981; Mathews and Roehrig, 1984; Mehra et al., 1986). Second approach used was by selecting escape variants against neutralizing Mabs, and sequence the variants in the region of the genome encoding E and M (Lobigs et al., 1987). It was found that each of the variant resulted from a single nt change in the E protein coding sequence leading to a nonconservative amino acid substitution at position 71 or 72 in the N-terminal region of E. Third approach made use of the λgt 11 expression system, which involves construction of expression libraries containing fragments of gene encoding the antigen, and sequence the cDNA expression clone that reacts with an antibody. The DNA sequence encoding the epitope is attributed to sequences that are shared by multiple antibody-positive recombinant clone (Mehra et al., 1986). We developed another useful approach for mapping the linear antigenic determinant of E protein of DEN-2 recognized by a type-specific and neutralizing Mab, 3H5, which has been shown in previous studies to have a significantly high neutralization titer (Gentry et al., 1982).

4.0 Body of the Report

Experimental:

1. Neutralization of the infectivity of the virus by anti-3H5 peptide

In the previous annual report, we described the mapping of the neutralizing determinant of the DEN-2 E protein recognized by the monoclonal antibody 3H5. This region of the E protein lies between amino acids 386-397, QLKLNWFKKGSS. Antibodies raised against this peptide linked to KLH neutralized the infectivity of DEN-2 with a 50% titer of 1:40. We investigated whether this low neutralization titer is due to the peptide itself or the technique of immunization. Since we had raised the anti-peptide antibodies in only one rabbit, we repeated the immunization in another rabbit. The peptide was coupled to KLH as described previously. The coupling reagent m-maleimidobenzoylsulfosuccinimide ester (Pierce Chemical Co., Rockford Ill.) was used to attach the peptide, via N-terminal cysteine residue, to Keyhole limpet hemocyanin (Sigma)

according to the procedure of Liu and co-workers (1979); coupled peptide was stored at - 20^0 C for use.

2. Antibody Production

Antiserum to synthetic peptide-KLH conjugate was obtained from 10 week-old male New Zealand white rabbit. The animal was first inoculated intramuscularly with 200 µg peptide-KLH conjugate emulsified in Freund complete adjuvant (Difco Laboratories, Detroit, Mich.). On day 14, 100 µg peptide-KLH conjugate in 0.5 ml PBS and 0.5 ml Freund incomplete adjuvant was injected subcutaneously at multiple sites along the back. The rabbit was reinoculated four times at weekly intervals with the same amount used in the procedure for second inoculation. The rabbit was bled from ear vein 10 days after last injection.

3. Neutralization Test

Antiserum was inactivated at 56 C for 30 min. and serial 2-fold dilutions beginning at 1:10 were made. Appropriate dilutions of antiserum were incubated with 100 pfu of virus for 2 h. at room temperature. These mixtures were then assayed by plaque reduction neutralization test [Russell and Nisaluk, 1967]. Briefly, monolayers of CV1 cells were prepared by seeding $0.5x10^6$ cells in each well of 6-well tissue culture plates [Costar, Cambridge Mass.]. After overnight incubation, the growth medium was removed and each plate was inoculated with 0.3 ml antiserum - virus mixture. Adsorption of the virus inocula was carried out for 1 h at room temperature. After washing the monolayer of cells with MEM without serum, it was overlaid with 3 ml of MEM containing 0.9% agar. After the agar had solidified, the plates were incubated at 37 C in 5% CO_2 atmosphere. And the second overlay containing neutral red was added on the 6^{th} day. Plaques were counted on the 7^{th} day. Titers from plaque reduction neutralization test were expressed as mean percent plaque reduction (Table I).

4. Immunoprecipitation Assays

CV1 cells in T75 flasks were infected with DEN2 New Guinea C strain at approximately 1 pfu/cell. After 72 h post-infection, [^{35}S] methionine (10 μCi / ml) was added with methionine deficient medium for 3 h. The cells were disrupted by scraping, and cell pellets were obtained by low speed centrifugation. Cells were solubilized in RIPA buffer, containing 50 mM Tris, pH 7.5, 150 mM NaCl, 1% DOC, 0.5% NP40 and 0.1 mM PMSF, and sonicated for 1 min. The lysates were centrifuged at 100,000 x g for 1 h. Supernatants were mixed with appropriate antibodies and protein A agarose beads (Genzyme Corp., Boston, Mass.). The precipitates were washed, dissociated by boiling in sample buffer (Laemmli, 1970), and analysed by 10% SDS-PAGE. Gels were fixed, stained and processed for fluorography. Fig. 1 shows that a 60 kDa E glycoprotein was immunoprecipitated by the polyclonal mouse hyperimmune sera from the dengue-2 infected CV-1 cells, which was also reactive with the anti-3H5 peptide. These results confirm our mapping data that the neutralizing determinant of 3H5 monoclonal antibody being hydrophilic is exposed to the surface of the E protein and the antibodies against this determinant could react with the native protein.

5. Expression of NS1 in Mammalian Cells

a. Plasmid constructs

For the expression of full-length NS1, the pRP-2 clone was digested with BamHI (the site located in the C-terminal region of E), and SmaI (the site located in the polylinker region of pUC18 downstream from NS2A region) to release a fragment containing nt 1700-3760 of DEN-2 RNA (Irie et al., 1989). The plasmid pRSVneo containing the pRSV-LTR promoter and SV40 poly(A) site was digested with HindIII and SmaI, and the DNA was purified. A synthetic linker was made double-stranded by annealing of two

complementary oligonucleotides 5'-AGCTTCCACCATGG-3' and 3'-AGGTGGTACCCTAG-5' which contained the translational initiation codon within an NcoI site, as well as 5'-cohesive BamHI and HindIII sites. It was used to join the NS1 coding sequence and the vector DNA. The strategy is outlined in Fig. 2

b. Expression of NS1 in CV-1 cells

The plasmid vector used for the NS1 expression construct was the pRSVneo. It contains the RSV-LTR promoter, the SV40 small t intron and the polyadenylation signal, which are required for gene expression in mammalian cells. The NS1 coding sequence was cloned under the RSV-LTR control to replace the *neo* gene (Fig. 2). Mature NS1 in dengue virus-infected cells is synthesized by proteolytic cleavage of the polyprotein precursor. Therefore, for the expression of NS1 from a eukaryotic vector, the translation initiation codon needs to be present upstream to the signal sequence for targeting NS1 to ER, and subsequent processing to generate mature NS1 on the cell surface. The C terminal domain of E protein upstream to the NS1 in the polyprotein genetic map probably serves as a signal sequence for the insertion of the NS1 protein across the membrane and into the lumen of the endoplasmic reticulum, where it is cleaved by the host signal peptidase at the putative cleavage site to generate the mature NS1. The first ATG of the NS1 coding sequence was supplied by a synthetic Kozak consensus sequence to serve as an initiation codon for the synthesis of NS1. To examine the expression of NS1, the plasmid pRSV-NS1 was transfected into CV-1 cells by calcium phosphate-DNA precipitation method. The tranfected cells were checked for NS1 expression by indirect immunofluorescence using monoclonal antibody against DEN-2 NS1 (3E9) and the hyperimmune mouse ascites fluid (HMAF). The expression of NS1 was also monitored by immunoprecipitation. The transfected CV-1 cells were labeled with (^{35}S) methionine, 36 hrs post transfection. The cell lysate was immunoprecipitated with HMAF and 3E9 monoclonal antibody. Both methods failed to detect any expression of

NS1 (data not shown). The pRSV-β-gal was used as a control plasmid to monitor the efficiency of transfection by measuring the activity of β-galactosidase, and it was inferred that the failure of NS1 expression was not due to the poor efficiency of transfection, but it was either due to a problem inherent with the expression system, viz., nuclear transcription of the gene, transport of mRNA to the cytoplasm, and subsequent translation, or due to a mutation within the gene itself. Next, in order to test the latter possibility, the NS1 gene was cut out from the pRSV-NS1 construct and was cloned into an expression vector containing the T7 promoter and encephalomyocartitis (EMC) virus 5'-untranslated leader sequence. The unique feature of this expression system is that the large fraction of RNA molecules transcribed from the T7 promoter is translated via a cap-independent mechanism. For transcription of the NS1 gene, the T7 RNA polymerase was provided by infection of the transfected CV1 cells with a recombinant vaccinia virus. By metabolic labeling and radioimmuneprecipitation of the NS1-transfected cells, it was found that NS1 was expressed at a high level, indicating that there is no mutation in the coding sequence for NS1 (Dr. Padmanabhan, unpublished observations). Therefore, the failure of the pRSV-NS1 construct in CV1 cells may be due to an undesirable posttranscriptional processing of NS1 gene encoded by the pRSV-NS1.

6. Construction of Long cDNA Clones in Plasmid Vectors

a. Rationale

The complete nucleotide sequence of DEN-2 (NGS-C) has been reported previously (Irie et al, 1989). This report described the complete RNA genome assembled from several cDNA clones. Several other groups have been successful in making full length cDNA clones of RNA viruses that could be propagated in bacterial plasmids in some cases, whereas in the case of YF virus, it was assembled *in vitro* due to its instability in E. coli (Rice et al. 1989). We used this approach to construct a plasmid DNA that contained

all the viral structural proteins by series of ligation steps from several overlapping cDNA clones. The virion structural proteins are encoded in the 5' portion of the genome and the gene order is:

5'-C-prM-(M)-E-NS1-NS2A-NS2B-NS3-NS4A-NS4B-NS5-3.

Mature virions consist of two transmembrane proteins, E, the envelope protein, and M, the membrane protein. The E protein, which contains the neutralization epitopes of the virus, has been the subject of intensive structural and immunological characterization (for a review, see Heinz, 1986). The future goal of our laboratory is to construct the full length cDNA clone for DEN-2 in order to study the expression and processing of the polyprotein to give rise to mature viral proteins, and to study their interaction to form the virus particle.

b.Strategy for the construction of long DEN-2 cDNA clone.

The available cDNA clones, pKT 2.4 (Irie et al, 1989), pRP 2 (Putnak et al, 1988), and pKT 89 (Irie et al, 1989) contain the sequences that code for structural proteins C, prM(M), and E, as well as nonstructural proteins NS1, NS2A, NS2B, and the N-terminal portion of NS3. The longest DEN-2 cDNA clone (about 2.4 kb from 7 nt- nt 2340) in the plasmid pKT 2.4 was originally cloned at the EcoRI site of pUC18 vector. The EcoRI fragment from pKT 2.4 was transferred to the EcoRI site of plasmid vector pGem7Zf(+). This plasmid was then partially digested with EcoRI, and the DNA was purified by electrophoresis on agarose. The DNA band (5.4 kb) was cut out and purified. This linear band was ligated to the EcoRI fragment isolated from the pRP-2 clone (535 bp; nt2340-2871). The orientation of the 535 bp insert was verified by restriction analysis. The plasmid containing 2871 bp of DEN-2 sequence (pTT 36) was partially digested with EcoRI and separated on agarose, and the largest band was cut out and purified. This linear fragment (6 kb) was used as a vector for cloning the EcoRI fragment from the clone pKT 89/pUC18 cDNA clone (nt~3050-4700). The desired clone was identified by restriction

analysis of the transformants resulting from insertions of DNA at three EcoRI sites in two orientations. The final clone pTT 47 contains 4.7 kb of DEN-2 cDNA at the EcoRI site of pGem7Zf(+) under T7 promoter. The cloning strategy is shown in Fig. 3.

c. Cloning long cDNA by ligation in vitro

With the overall goal of constructing a full-length cDNA for DEN-2, a cDNA clone starting from + 7 in the 5'-noncoding region and ending at nt 4700 of the DEN-2 genome was assembled to give rise to the plasmid pTT 47. The long cDNA clone pTT 47 contains 4.7 kb of DEN-2 sequence that encodes all the structural proteins and the nonstructural proteins NS1, NS2A, NS2B and the N-terminal portion of NS3. It is missing about 180 nucleotides from nt 2872 to nt 3050 of DEN-2 genome due to the choice of pKT89 clone in the construction of pTT47. This missing region is between two unique restriction sites (XmnI and BstXI), and can be readily repaired by ligating the intact XmnI-BstXI fragment from the pRP-2 cDNA clone to replace the XmnI-BstXI fragment of clone pTT47. However, *E.coli* cells harboring this pTT47 plasmid grew poorly. Other laboratories have encountered similar problems with cDNA clones of similar length from other flaviviruses (Rice et al, 1989). This cDNA clone was, however, useful as a template for the PCR amplification to isolate the complete coding sequence of E protein, and its expression in a baculovirus system (see below).

7. Baculovirus expression

a. Rationale: The baculovirus expression system offers a unique advantage in view of the high levels of foreign gene expression obtainable with the viral polyhedrin promoter (Luckow and Summers, 1988). One of the major advantages of this invertebrate virus expression vector over bacterial, yeast and mammalian expression systems is the very abundant expression of recombinant proteins, which in many cases, maintain the antigenicities, immunogenicities and functionalities of the native proteins. In

addition, some of the protein modifications, such as targeting to the nucleus and the endoplasmic reticulum (ER), glycosylation, secretion, the processing of precursor to mature proteins that normally occur in higher eukaryotic cells do occur in insect cells. These modifications may be essential for the complete biological function of a recombinant protein. The genome of *Autographa californica* nuclear polyhedrosis virus (AcMNPV), the prototype of the Baculoviridae family, consists of double-stranded, circular, supercoiled DNA of approximately 128 kilobases in length. During AcMNPV infection, two forms of viral progeny are produced: extracellular virus particles (ECV), and occluded virus particles (OV). The latter are embedded in proteinaceous viral occlusions, consisting of a major viral encoded 29 kDa structural protein, the polyhedrin. In infected *Spodoptera frugiperda* cell cultures, polyhedra accumulate to very high levels, routinely 1 mg/ml per 1.0-2.0x10^6 infected cells, accounting for about 50% of the total proteins synthesized. Since the polyhedrin synthesis occurs at a very late phase of the virus life cycle, even protein that are "toxic" to cells are synthesized at high levels in this system. The polyhedrin gene under the control of the powerful late promoter is not essential for viral DNA replication. The gene is cloned under the control of polyhedrin promoter at a site flanked by AcMNPV sequences to facilitate homologous recombination and insertion of the foreign gene into the wild type genomen resulting in the insertional inactivation of the polyhedrin gene. The resulting recombinant virus exhibit occlusion negative (Occ^-) phenotype, which form plaques that are distinguishable from those of wild-type (Occ^+) viruses. These distinctive plaque morphologies of the recombinant viruses, in which the wild-type AcMNPV polyhedrin gene has been replaced with the gene of interest, provide a way to visually screen for recombinant viruses under a light microscope. The nonessential nature and high levels of expression of the polyhedrin gene, and the relative ease with which recombinant Occ^- viruses can be detected make this expression system an attractive one.

b. Development of a new transfer vector: The most widely used transfer vectors for introducing foreign genes into wild-type baculovirus are pAc373 (Smith et al., 1985), pE-55 (Miller et al., 1986), pAcYM1 (Matsuura et al., 1987). These vectors contain the 5'- and 3'-franking regions of the polyhedrin gene, the polyhedrin promoter and polyadenylation site, and plasmid pUC8 (amp^r) sequences that includes *ori* to allow their amplification in E. coli. These plasmid vectors differ in the nature of the restriction sites available for cloning, and the expressed foreign proteins as fused or non-fused products. The viral polyhedrin gene contain a 5' untranslated AT-rich leader sequence (approximately 50 bp), which may be necessary for abundant expression of this late gene product. Any mutation in this region decreases protein expression significantly (Miller, 1988). The available cloning site in one vector is Bam HI, which is 177 bases downstream of the polyhedrin initiation codon. Any foreign gene cloned into this site will be expressed as a fusion protein linked to several N-terminal amino acids of polyhedrin. In order to express a protein in a non-fused form, the polyhedrin coding sequence upstream of this Bam HI site has to be removed. The common strategy previously used to achieve this goal was to digest the BamHI-linearized vector with Bal 31 (Smith et al., 1983) to remove almost all of 177 bp of the polyhedrin coding sequences, followed by ligation to a BamHI linker. This strategy resulted in an expression plasmid pAc373 (Smith et al., 1983, 1985) in which eight nt of the 5'-leader sequences were also removed, as it was difficult to control the Bal 31 digestion. Our laboratory devised a novel strategy to construct a transfer vector, in which the 105 bp polyhedrin promoter containing all of the 5'-leader sequences, but lacking an initiation codon was constructed from four overlapping synthetic oligomers. Annealing these oligomers, and gap-filling with the Klenow DNA polymerase I, followed by cloning the 105 bp polyhedrin promoter fragment into pUC18 vector resulted in an intermediate transfer vector suitable for cloning the foreign gene of interest at the unique BamHI site. The foreign gene to be expressed was then excised from this

intermediate vector, and cloned into the final transfer vector containing more AcMNPV flanking sequences in order to facilitate *in vivo* recombination into AcmNPV genome. This transfer vector was used to express E. coli β–galactosidase, as well as adenovirus preterminal protein (Zhao, Irie, and Padmanabhan, unpublished results).

Screening for the recombinant baculovirus, and plaque-purifying the virus free of contaminating wild-type virus can be very tedious. In order to facilitate the screening process, we modified the transfer vector which is described above as follows. It is known for some time that coexpression of E. coli β-galactosidase and the gene of interest off of a vaccinia virus expression vector allowed the visual selection of recombinant vaccinia virus plaques in the presence of a chromogenic substrate for the β-galactosidase enzyme (Chakraberty et al., 1985). Hence, it would be very useful to develop a similar feature for the baculovirus expression system. we modified the transfer vector to include the E. coli β-galactosidase gene under the control of Rous sarcoma virus long terminal repeat (RSV-LTR) promoter upstream from the polyhedrin promoter. The modified vector was shown to be functional in *in vivo* recombination in insect cells, giving rise to blue plaques due to the expression of the E. coli β-galactosidase and its reaction with a chromogenic substrate.

c. Construction of a Baculovirus Transfer Vector containing Two Promoters and the β-Galactosidase Gene.

A baculovirus vector was designed to incorporate features of high level protein expression as well as rapid screening of recombinant virus The pEI vector was modified as outlined in Fig.4. The additions made to this plasmid are described in detail in Materials and Methods. Briefly, a transcription unit containing RSV-LTR promoter and the coding sequence for β-galactosidase was cloned into Eco RV site of pKX105 (Zhao et al., submitted for publication) upstream from polyhedrin promoter in opposite direction. During the cloning and screening step, transformed HB101 were plated on LB-Xgal plate with ampicillin. The blue colonies were selected and the DNA from independent clones

were analyzed using restriction enzymes to distinguish the two possible orientations of the transformants.The recombinant plasmids that contain b-gal gene in the right orientation (pKX-β-gal) was amplified. The XhoI and BamHI DNA fragment from pKX-β-gal was directionally cloned into transplacement vector pEI (pEI-β-gal)

Our initial experiments were designed to determine whether the E.coli β-gal gene placed under the control of RSV promoter could be expressed in insect cells when cotransfected with wild type virus. The β-gal gene was flanked by homologous segments of AcNPV genome, so that both the marker gene and the foreign gene would be inserted into the baculovirus genome by homologous recombination.

To transfer pEI-β-gal into AcMNPV genome, the SF21 monolayer cells were cotransfected with AcMNPV DNA and pEI-β-gal DNA by a transfection technique based on liposome-mediated delivery of DNA. Recombinant virus isolates expressing the β-gal gene were detected as blue-colored plaques in the presence of the chromogenic substrates for β-gal, Xgal or Bluo-Gal added to the the agarose overlay medium. The Xgal stained monolayer plates showed that approximately 0.1-1% of the plaques expressed β-galactosidase after cotransfection of AcMNPV and the plasmid pEI-β-gal indicating the efficiency of in vivo recombination. Several blue plaques were picked and amplified by infection of fresh SF-21 cell monolayers.

d. Expression of E-protein in insect cells infected with a baculovirus recombinant.

d.1. PCR Amplification of E Coding Sequence

Two oligonucleotide primers were synthesized, PCR01, 5'-CATAATGGCAGCAATCCTGGCATAC-3' (nt 840-864 of DEN-2 genome), primer PCR02, 5'-GGCCTGCACCATAACTCCC-3' (nt 2421-2342 corresponding to the C-terminal sequence of E-protein. pTT 36 or pTT47 was digested by PstI, and the 2338 bp PstI fragment (nt 395-2733) was used as a template in PCR amplification. After

amplification, the PCR product was analyzed by gel electrophoresis and restriction analysis.

d. 2. Plasmid construct for expression of E-protein

Vector DNA pEI-β-gal was digested with BamHI and 3' protuding ends were filled in with Klenow DNA polymerase I. The fragment containing the E coding sequence obtained by PCR amplification as described above, was phosphorylated by polynucleotide kinase and ligated to pEI-β-gal vector. After transformation, the blue colonies were picked and screened by restriction analysis. The plasmid clone with correct orientation was purified.

d. 3. Expression of recombinant E protein in SF21 cells

The recombinant plasmid DNA (containing the coding sequence of E protein and the marker gene for β-gal under the control of two separate promoters), and the wild-type AcMNPV DNA were cotransfected into SF-21 cells to generate the recombinant baculovirus through homologous recombination. Recombinant baculovirus was selected as a blue plaque, followed by three rounds of plaque purification. The recombinant virus appeared to be stable during several rounds of plaque purification. Western blot analysis was used to detect the synthesis of E protein in the recombinant baculovirus-infected SF21 cells. Monoclonal antibody 3H5, specific for DEN-2 E protein and the polyclonal hyperimmune mouse ascites fluid were used to detect the expression of E protein. When the lysates from the recombinant virus-infected, wild-type baculovirus infected, and uninfected SF21 cells were analysed with these specific antibodies, only the lysate from the recombinant virus-infected cells expressing the E antigen reacted with each of these antibodies. The immuno-reactive band had a mobility slower than that of E protein produced during dengue virus infection in CV-1 cells (data not shown). This observation indicated that the coding sequence of E from the PCR-amplified DNA was transcribed, the resulting mRNA was translated, and may not properly processed to produce mature E

protein in insect cells. The recombinant protein appeared to be synthesized starting at 12 h. post infection. After 24 h. post infection the protein migrated at 55-60 kDa was partially cleaved or degraded to low molecular weight mass of about 50-55 kDa and 20 kDa . This 20 kDa protein seem to be increased with time (data not shown). This results indicated that E protein is not stable in the insect cells.

The level of E protein expressed in recombinant baculovirus-infected cells was compared with that produced in the dengue virus-infected CV-1 cells by Western blotting using monoclonal antibody 3H5. Western blot analysis showed that the amount of E produced in insect cells was lower of that produced in dengue virus infected cells at a multiplicity of infection of 1. The level of expression was also analysed by Coomassie blue staining of SDS/polyacrylamide gel electrophoresis of infected cell lysates (data not shown) A band of E protein was not seen at mobility of 55-60 kDa of protein. The predominant band was seen at mobility of 20 kDa which is corresponding to the predominant band in Western blot. The level of protein expression was not impressive, since E protein could barely be detected on SDS/polyacrylamide gel stained with Coomassie blue.

5.0 Conclusions:

1. It is confirmed now that a short peptide containing the sequence QLKLNWFKKGSS can elicit neutralizing antibodies in rabbits. This represents a region of DEN-2 E protein which is at least partly recognized by the neutralizing monoclonal antibody 3H5 from the binding data of the peptide with 3H5.

2. The anti-peptide antibody raised in rabbits can neutralize the infectivity of the DEN-2 with a 50% reduction of infectivity titer of 1:320. This titer is higher than the one obtainined from rabbit #1 (see 1989 annual report) which was 1:40. We think that the

reason for the low titer during the first attempt might be due to poor emulsion formed between the peptide-KLH-adjuvant conjugate used for immunization.

3. Attempt to express NS1 in mammalian cells using RSV-LTR promoter was not successful. The reason for the failure might be due to undesirable post-transcriptional processing taking place in the nucleus of the transfected cells. The possibility that there could be a nonsense mutation in the coding sequence of NS1 was ruled out by expression of NS1 under the control of T7 promoter in the cytoplasm.

4. Attempt to express E protein in insect cells using a baculovirus vector developed in our laboratory was also not successful. The same vector was used for the expression of other antigens suggesting that the construction of the vector was correct. The failure of E expression might be due to a defect in the signal sequence designed for targeting the protein to the ER, resulting in degradation of the protein.

5. A long cDNA from nucleotide 7 to 4700 of dengue genome was constructed. The bacteria harboring this clone seems to be unstable indicating that the cDNA insert might be toxic to the host.

6.0 References

Bancroft, W.H., Scott, R.M., Eckels, K.H., Hoke, C.H., Simms, T.E., Jesrani. K.D.T., Summers, P.L., Dubois, D.R., Tsoulos, D., and Russell, P.K. (1984). Dengue virus type 2 vaccine: Reactogenecity and immunogenicity in soldiers. J. Infect. Diseases 149, 1005-1010.

Berzofsky, J.A. (1985). Intrinsic and extrinsic factors in protein antigenic structure. Science. 229, 932-940.

Benjamin, D.C., Berzofsky, J.A., East, I.J., Gurd, F.R.N., Hannum, C., Leach, S.J., Margoliasch, E., Michael, J.G., Miller, A., Prager, E.M., Reichlio, M., Sercatz, E.E.,

Smith-Gill, S.J., Todd, P.E., and A.C. Wilson. (1984). The antigenic structure of proteins: a reappraisal. Annu. Rev. Biochem. 2, 67-101.

Bray, M., Falgout, B., Zhao, B., Markoff, L., Moss, B., Chanock, R.M., and Lai, C.-J (1989). Mice immunized with recombinant vaccinia virus expressing dengue 4 structural proteins and/or nonstructural protein NS1 are protected against fatal dengue encephalitis. In, "Vaccines 89-Modern Approaches to New Vaccines Including the Prevention of AIDS". Eds. R.A. Lerner, H. Ginsberg, R.M. Chanock and F. Brown. Cold Spring Harbor Press, Cold Spring Harbor, N.Y. pp. 357-362.

Castle, E., Leidner, U., Novak, T., Wengler, G. and Wengler, G.: Primary structure of the West Nile flavivirus genome coding for all non-structural proteins. Virology 149 (1986) 10-26.

Castle, E., Novak, T., Leidner, U., Wengler, G. and Wengler, G.: Sequence analysis of West Nile virus and of the genome sequence for these proteins. Virology 145 (1985) 227-236.

Chakrabarti, S., Brechling, K., and Moss, B. (1985). Vaccinia virus expression vector: Coexpression of b-galactosidase provides visual screening of recombinant virus plaque. Mol. Cell. Biol. 5,3403-3409.

Coia, G., Parker, M.D., Speight, G., Byrne, M.E. and Westaway, E.G.: Nucleotide and complete amino acid sequence of Kunjin virus: definitive gene order and characteristics of the virus-specified proteins. J. Gen. Virol. 69 (1988) 1-21.

Della-Porta, A.J., and Westaway, E.G. (1977). Immune response in rabbits to virion and non-virion antigens of the flavivirus Kunjin. Infect. Immun. 15, 874-882.

Duebel, V., Kinney, R.M. and Trent, D.W.: Nucleotide sequence and deduced amino acid sequence of the nonstructural proteins of dengue type 2 virus, Jamaica genotype. comparative analysis of the full-length genome. Virology 165 (1988a) 234-244.

Deubel, V., Kinney, R.M., Esposito, J.J., Cropp, C.B., Vorndam, A.V., Monath, T.P., and Trent, D.W. (1988b). Dengue 2 virus envelope protein expressed by a recombinant vaccinia virus fails to protect monkeys against dengue. J. Gen. Virol. 69, 1921-1929.

Deubel, V., Kinney, R.M. and Trent, D.W.: Nucleotide sequence and deduced amino acid sequence of the structural proteins of dengue type 2 virus, Jamaica genotype. Virology 155 (1986) 365-377.

Eckels, K.H., Scott, R.M., Esposito, J.J., Brown, J., Dubois, D.R., Summers, P.L., Russell, P.K., and Halstead, S.B. (1984). Selection of attenuated dengue 4 viruses by serial passage in primary kidney cells. Am J. Trop. Med. Hyg. 33, 684-689.

Emini, E.A., Jameson, B.A., Lewis, A.J., Larsen, G.R., and Wimmer, E. (1982). Poliovirus neutralization epitopes: analysis and localization with neutralizing monoclonal antibodies. J. Virol. 43, 997-1005.

Falgout, B., Chanock, R., and Lai, C.-J. (1989). Proper processing of dengue virus nonstructural glycoprotein NS1 requires the N-terminal hydrophobic signal sequence and the downstream nonstructural protein NS2A. J. Virol. 63, 1852-1860.

Gentry, M.K., Henchal, E.A., McCown, J.M., Brandt, W.E., and Dalrymple, J.M. (1982). Identification of distinct antigenic determinants on dengue-2 virus using monoclonal antibodies. Am J. Trop. Med. Hyg. 31, 548-555.

Halstead, S.B. : Pathogenesis of dengue: Challanges to molecular biology. Science 239 (1988) 476-481.

Hahn, Y.S., Galler, R., Hunkapillar, T., Dalrymple, J.M., Strauss, J.H. and Strauss, E.G. : Nucleotide sequence of dengue 2 RNA and comparison of the encoded proteins with those of other flaviviruses. Virology 162 (1988) 167-180.

Heinz, F.X., Berger, R., Tuma, W., and Kunz, C. (1983). A topological and functional model of epitopes on the structural glycoprotein of tick-borne encephalitis virus defined by monoclonal antibodies.

Heinz, F.X., Tuma, W., and Kunz, C. (1981). Antigenic and immunological properties of defined physical forms of tick-borne encephalitis virus structural proteins. Infect. and Immun. 33, 250-257.

Heinz, F.X., Berger, R., Tuma, W., and Kunz, C. (1983a). A topological and functional model of epitopes on the structural glycoprotein of tick-borne encephalitis virus defined by monoclonal antibodies. Virology 126, 525-537.

Heinz, F.X., Berger, R., Tuma, W., and Kunz, C. (1983b). Location of immunodominant antigenic determinants on fragments of the tick-borne encephalitis virus glycoprotein: evidence for two different mechanisms by which antibodies mediate neutralization and hemagglutination inhibition. Virology 130, 485-501.

Heinz, F.X. (1986). Epitope mapping of flavivirus glycoproteins. Virus Res. 31, 103-186.

Irie, K., Mohan, P.M., Sasaguri, Y., Putnak, R., and Padmanabhan, R.: Sequence analysis of cloned dengue virus type 2 genome (New Guinea-C strain). Gene 75 (1989) 197-211.

Kitano, T., Suzuki, K., and Yamaguchi, T. (1974). Morphological, chemical, and biological characterization of Japanese encephalitis virus virion and its hemagglutinin. J. Virol. 14, 631-639.

Lai, C.J., Zhang, Y.M., Bray, M., Chanock, R., and Eckels K.H. (1989). Use of dengue virus structural proteins and nonstructural protein NS1 produced by recombinant baculovirus for immunization against dengue virus infection. In, "Vaccines 89-Modern Approaches to New Vaccines Including the Prevention of AIDS". Eds. R.A. Lerner, H. Ginsberg, R.M. Chanock and F. Brown. Cold Spring Harbor Press, Cold Spring Harbor, N.Y. pp. 1-6.

Liu, F. -T., Zinecker, M., Hamaoka, T., and Katz, D.H. (1979). New procedures for preparation and isolation of conjugates of proteins and synthetic co-polymer of D- amino acids and immunochemical characterization of such conjugates. Biochemistry 18:690-697.

Lobigs, M., Dalgarno, L., Schlesinger, J.J., and Weir, R.C. (1987). Location of a neutralization determinant in the epitope of yellow fever virus (17D vaccine strain). Virology 161, 474-478.

Luclow, V.A. and Summers, M.D.: Trends in the development of baculovirus expression vectors. Bio/Technology 6 (1988) 47-55.

Lubeck, M.D., and Gerhard, W. (1982). Conformational changes at topologically distinct antigenic sites on the influenza A/PR/8/34 molecule are induced by the binding of monoclonal antibodies. Virology 118, 1-7.

Mackow, E., Makino, Y., Zhao, B., Zhang, Y-M., Markoff, L., Buckler-White, A., Guiler, M., Chanock, R. and Lai, C-j. : The nucleotide sequence of dengue type 4 virus: analysis of genes coding for nonstructural proteins. Virology 159 (1987) 217-228.

Mason, P.W., McAda, P.C., Mason, T.L., Fournier, M.J. (1987). Sequence of the dengue virus genome in the region encoding the three structural proteins and the major nonstructural protein NS1. Virology. 161, 262-267.

Massey, R.J., and Schochetman, G. (1981). Topographical analysis of viral epitopes using monoclonal antibodies: mechanism of virus neutralization. Virology 115, 20-32.

Matsuura, Y., Posse, R.D., Overton, H.A., and Bishop, D.H.L.: Baculovirus expression vectors: the requirements for high level expression of proteins including glycoproteins. J. Gen. Virol. 68 (1987) 1233-1250.

Mehra, V., Sweetser, D., and Young, R.A. (1986). Efficient mapping of protein antigenic determinants. Proc. Nat. Acad. Sci. USA 83, 7013-7017.

Miller, D.W., Safer, P., and Miller, L.K.: An insect baculovirus host-vectorfor high level expression of foreign genes. In Setlow, J.K., and Hollander, A. (Eds.), Genetic Engineering. Plenum Press, New York, 1986, vol. 8, pp. 277-298.

Putnak, R., Charles, P.C., Padmanabhan, R., Irie, K., Hoke, C., and Burke, D. S. (1988). Functional and antigenic domains of the dengue-2 virus non-structural glycoprotein NS1. Virology 163, 93-103.

Rice, C.M., Lenches, E.M., Eddy, S.R., Shin, S.J., Sheets, R.L. and Strauss, J.H. : Nucleotide sequence of yellow fever virus: Implications for flavivirus gene expression and evolution. . Science 229 (1985) 726-733.

Rice, C.M., Grakoui, A., Galler, R., and Chambers, T.J. (1989). Transcription of infectious yellow fever RNA from full-length cDNA templates produced by in vitro ligation. The New Biologist 1, 285-296.

Roehrig, J.T., Day, J.W., and Kinney, R.M. (1982). Antigenic analysis of the surface glycoproteins of a Venezuelan equine encephalomyelitis virus (TC-83) using monoclonal antibodies. Virology 118, 269-278.

Russell, P.K. and Nisalak, A. (1967). Dengue virus identification by the plaque reduction neutralization test J. Immunol. 99, 291-296.

Russell, P.K., Brandt, W.E. and Dalrymple, J.M.: Chemical and antigenic structure of flaviviruses. In Schlesinger, R.W. (Ed.), The Togaviruses. Academic Press, New York, 1980, pp. 503-530.

Schlesinger, J.J., Brandriss, M.W., and Walsh, E.E. (1985). Protection against 17D yellow fever encephalitis in mice by passive transfer of monoclonal antibodies to the non-structural glycoprotein gp48 and by active immunization with gp48. J. Immunol. 135, 2805-2809.

Schlesinger, J.J., Brandriss, M.W., and Walsh, E.E. (1987). Protection of mice against dengue 2 virus encephalitis by immunization with dengue 2 virus non-structural glycoprotein NS1. J. Gen. Virol. 68, 853-857.

Smith, G.E., Fraser, M.J., Summers, M.D. (1983). Molecular engineering of the Autographa californica nuclear polyhedrosis virus genome: deletion mutations within the polyhedrin gene. J. Virol. 46, 584-593.

Smith, G.E. Ju, G., Ericson, B.L., Moschera, J., Lahm, H., Chizzonite, R., and Summer, M.D. (1985). Modification and secretion of human interleukin 2 produced in insect cells by a baculovirus expression vector. Proc. Natl. Acad. Sci. USA 82,8404-8408.

Speight, G., Coia, G., Parker, M.D., and Westaway, E.G.: Gene mapping and positive identification of the nonstructural proteins NS2A, NS2B, NS3, NS4B and NS5 of the flavivirus Kunjin and their cleavage sites. J. Gen. Virol. 69 (1988) 23-34.

Sumiyoshi, H., Mori, C., Fuke, I., Morita, K., Kuhara, S., Kondou, J., Kikuchi, Y., Nagamatu, H. and Igarashi, A.: Complete nucleotide sequence of Japanese encephalitis virus genome RNA. Virology 161 (1987) 497-510.

Volk, W.A., Snyder, R.M., Benjamin, D.C., and Wagner, R.R. (1982). Monocional antibodies to the glycoprotein of vesicular stomatitis virus: comparative neutralizing activity. J. Virol. 42, 220-227.

Westaway, E.G., Brinton, M. A., Gaidamovich, S.Y., Horzinek, M.C., Igarashi, A., Kaariainen, L., Lvov, D.K., Porterfield, J.S., Russell, P.K. and Trent, D.W. : Flaviviridae. Intervirology. 24 (1985) 183-192.

Yaegashi, T., Vakharia, V.N., Page, K., Sasaguri, Y., Feighny, R. and Padmanabhan, R.: Partial sequence analysis of cloned dengue virus type 2 genome. Gene 46 (1986) 257-267.

Zhang, Y-M., Hayes, E.P., McCarty, T.C., Dubois, D. R., Summers, P.L., Eckels, K.H., Chanock, R.M., and Lai, C.-J. (1988). Immunization of mice with dengue structural proteins and nonstructural protein NS1 expressed by baculovirus recombinant induces resistance to dengue virus encephalitis. J. Virol. 62, 3027-3031.

Zhao, B., Mackow, E., Buckler-White, A., Markoff, L., Chanock, R.M., Lai, C-J. and Makino, M.: Cloning full-length dengue type 4 viral DNA sequences: analysis of genes coding for structural proteins. Virology 155 (1986) 77-88.

Zhao, B., Prince, G., Horswood, R., Eckels, K.H., Summers, P.L., Chanock, R.M., and C.-J. Lai. (1987). Expression of dengue virus structural proteins and nonstructural protein NS1 by a recombinant vaccinia virus. J. Virol. 61, 4019-4022.

7.0 Appendix

7.1 Figure Legends

Fig. 1. Immunoprecipitation of DEN-2 E glycoprotein with anti-3H5 peptide antibody

CV-1 cells were infected with DEN-2 virus at a moi of 1, and were labeled with [^{35}S] methionine. Soluble cell lysates from uninfected (lanes 1, 3, and 5), and DEN-2-infected (lanes 2, 4, 6 and 7) CV-1 cells were reacted with the following antibodies : HMAF (lanes 1 and 2); 3H5 monoclonal antibody (lanes 3 and 4); rabbit antiserum to the synthetic peptide (lanes 5 and 6); rabbit preimmune serum (lane 7). Details of radiolabelling, immunoprecipitation, and analysis by SDS/PAGE are described in Methods. The mobilities of molecular weight markers are indicated.

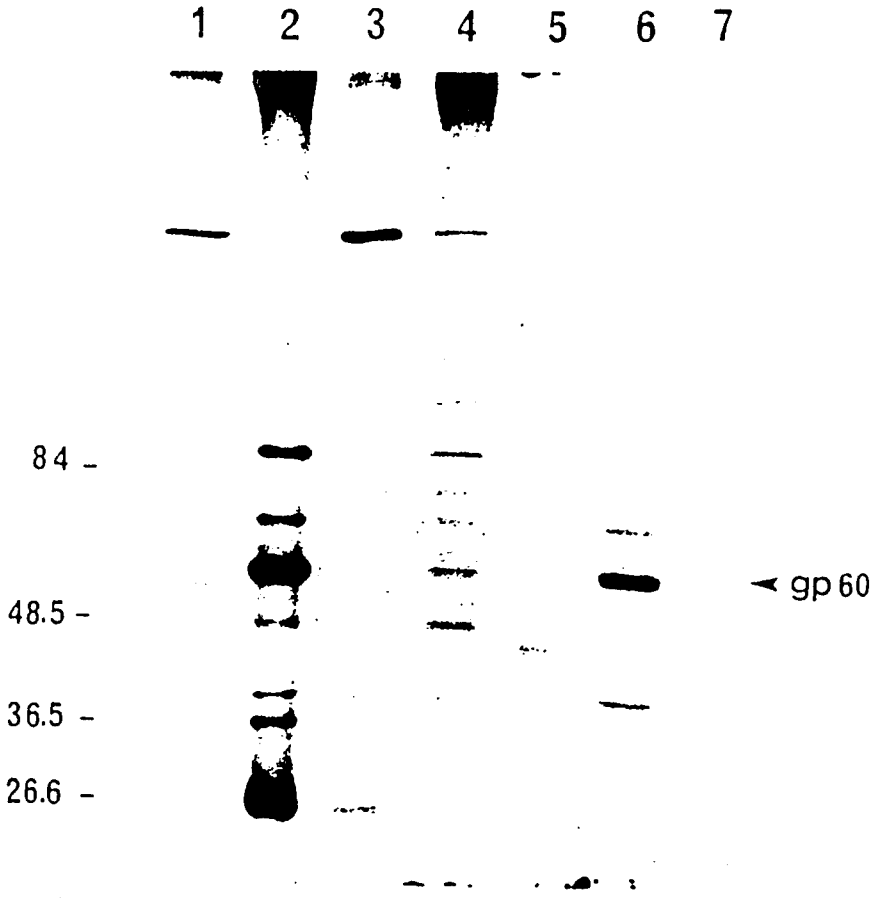
Fig. 2. Strategy for cloning long cDNA in plasmid vector.

The long cDNA coding for all structural proteins and some nonstructural proteins of DEN-2 virus were cloned by *in vitro* ligation of 3 overlapping clones, pKT 2.4 (Irie et al., 1989) pRP-2 (Putnak et al., 1988) and pKT 89 (Irie et al., 1989). By using a series of partial digestions with Eco RI and subsequent ligation as described in Methods, the final clone pTT 47 was obtained. Plasmid pTT47 contains 4.7 Kb of DEN-2 sequence that encodes all the structural proteins and the nonstructural proteins NS1, NS2A, NS2B, and the N-terminal portion of NS3. This 4.7 Kb of DEN-2 sequence was cloned into the Eco RI site in the multiple cloning site of pGem7Zf(+) under the control of the T7 promoter.

Fig. 3. The Modified Baculovirus Vector.

The pKX vector contains the XhoI-KpnI fragment of the AcMNPV genome cloned into the PstI-KpnI site of pUC18 vector, and the modified polyhedrin gene of baculovirus.

picked and screened by digestion with restriction enzymes to verify the orientation of the insert. The plasmid clone with the correct orientation was purified and used in cotransfection with AcMNPV into SF21 cells. The recombinant baculovirus plaques were picked and purified away from wild-type virus plaques by blue color.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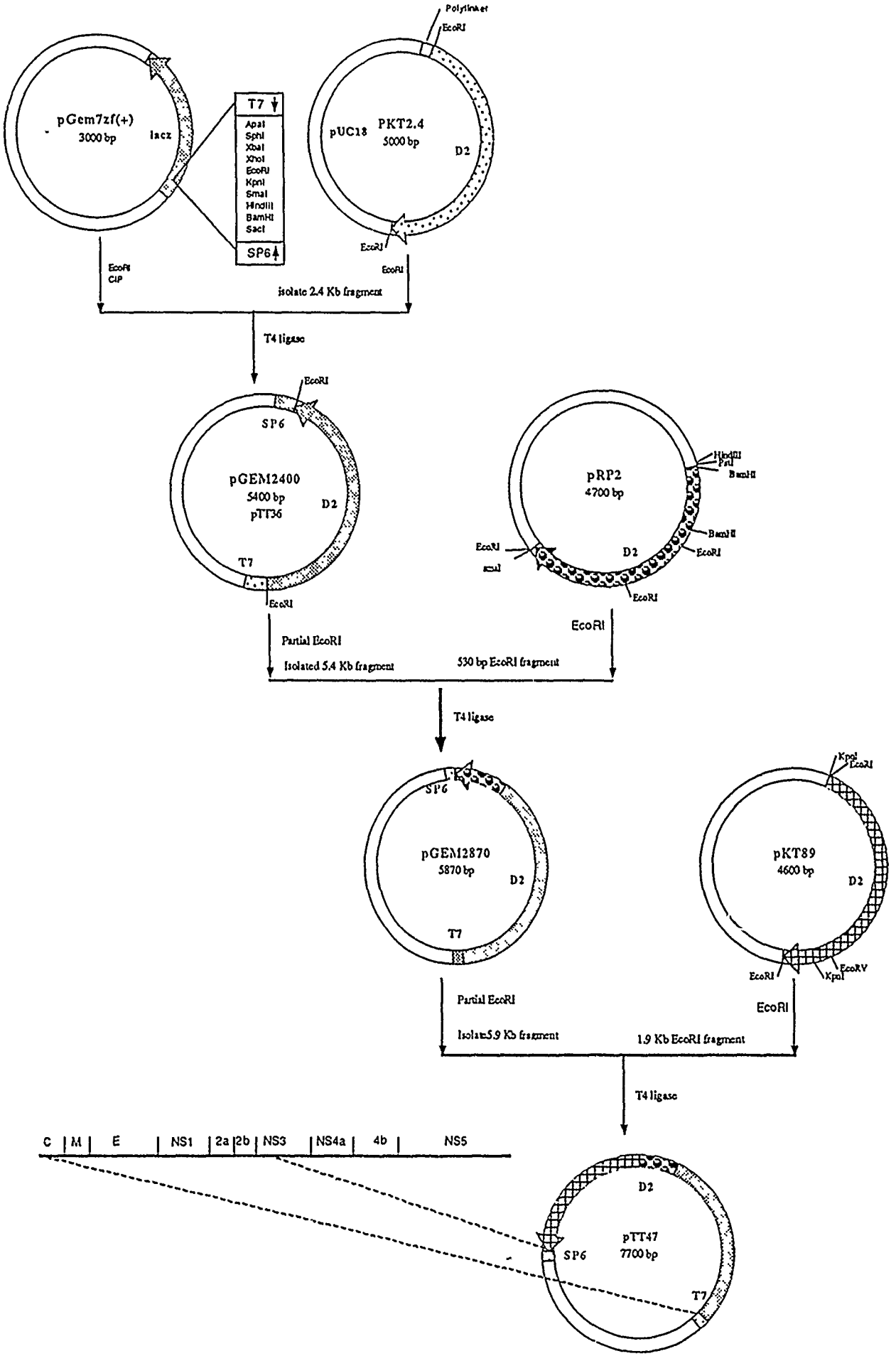
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Polylinker
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pGEM2400
5400 bp
pTT36
SP6
D2
T7
EcoRI
EcoRI
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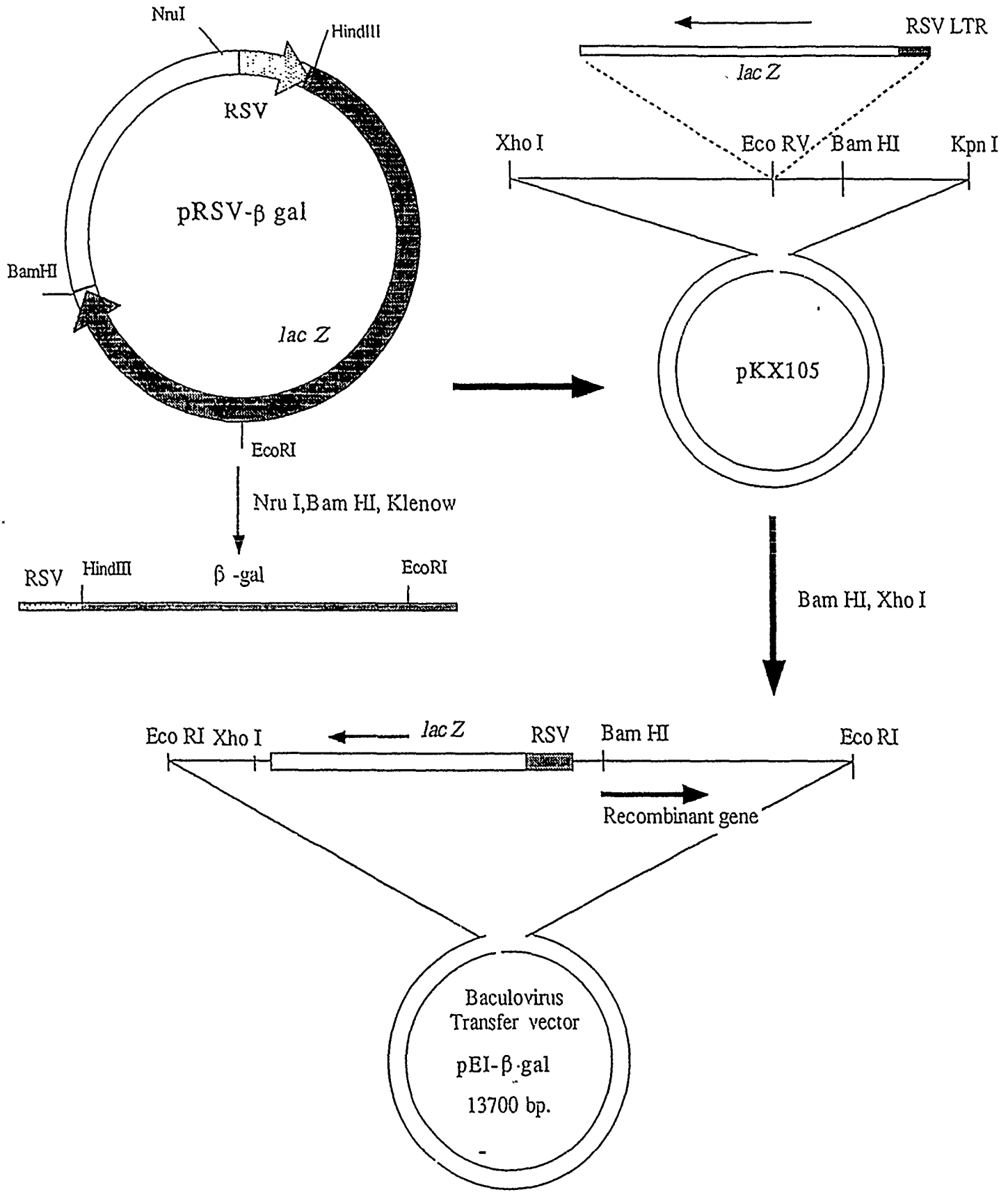
T7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lac Z
Xho I
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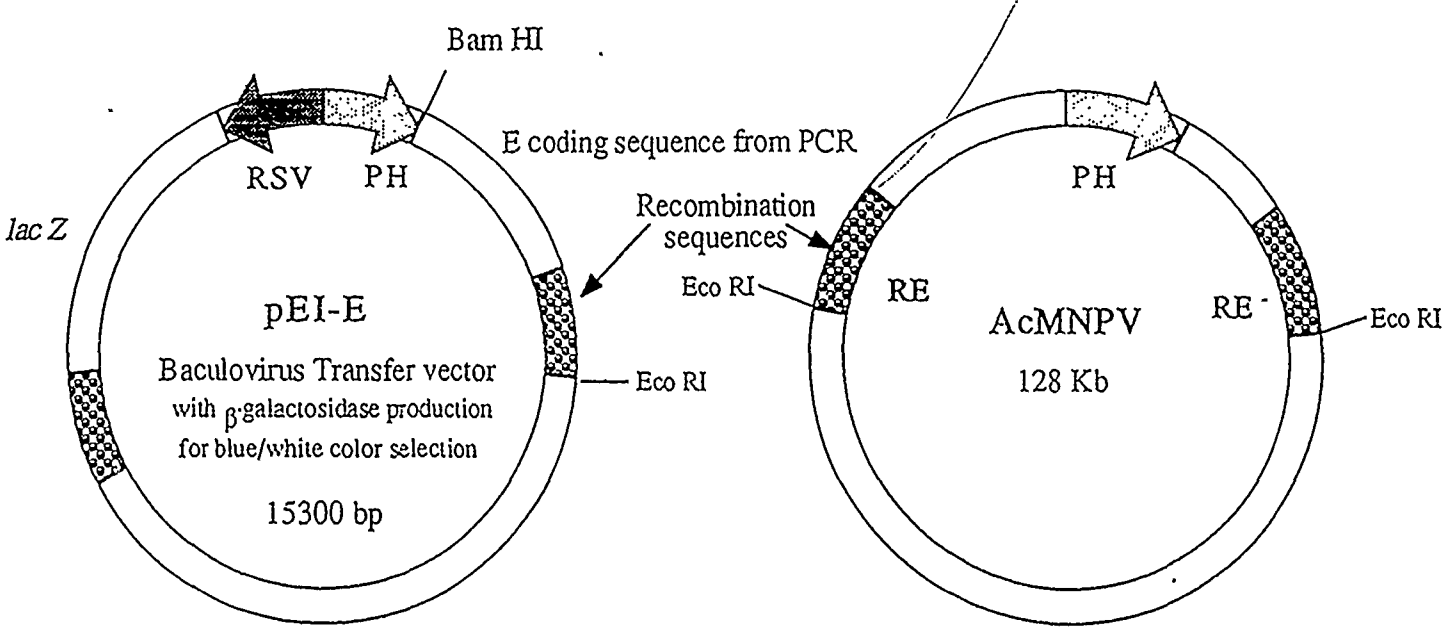

CO-TRANSFECTION

PLAQUE PURIFICATION OF BLUE RECOMBINANT BACULOVIRUS

INFECTION OF SF 21 CELLS AND PROTEIN PRODUCTION

PERSONNEL FUNDED BY DAMD17-85-C-5273 FROM SEPTEMBER 15, 1989 THROUGH SEPTEMBER 14, 1990

Thaweesak Trirawatanapong	Graduate student	50%	9/15/89- 7/20/90	10.2 mo.
Luwen Zhang	Graduate student	50%	9/15/89- 4/29/90 6/24/90- 9/14/90	8.2 mo
Keven Graham	Research Assistant	100%	10/01/89- 8/23/90	10.8 mo

PERSONNEL FUNDED BY DAMD17-85-C-5273 FROM SEPTEMBER 15, 1989 THROUGH SEPTEMBER 14, 1990

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